

FINAL REPORT

NASA GRANT NAG8-824

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**DEFINITION STUDY FOR TEMPERATURE CONTROL
IN ADVANCED PROTEIN CRYSTAL GROWTH**

Period of Performance
3/14/90 through 9/30/90

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1. Introduction

This Final Report summarizes the work performed during the funding period of NASA Grant NAG8-824, i.e. from March 14, 1990 through September 30, 1990. This research has been aimed at defining some of the technical requirements for an expedient application of temperature control to **Advanced Protein Crystal Growth** activities. In particular, in continuation of work performed under NASA Grant NAG8-711, useful ranges for temperature ramping and gradients were to be determined for various proteins.

We had originally proposed to study the temperature dependence of the growth and dissolution behavior of canavalin in its three crystal forms. However, our research following the submission of the proposal showed that the solubility of canavalin depends more on pH (which, in turn, changes even in buffered solutions) than on temperature. Although we actually succeeded in growing canavalin in the sting cell, it took a very long time for nucleation to occur and an even longer time to dissolve the crystals. Thus, obviously, canavalin is not a good candidate for demonstrating the advantages of temperature control. Consequently, we resumed our work with lysozyme in order to study the effects of temperature ramping and temperature gradients for nucleation/dissolution and consecutive growth of sizable crystals and, to determine a prototype temperature program.

We had also proposed to utilize the scintillation technique for solubility determinations developed under the foregoing grant and to semi-automate solubility measurements through optimization of the computer control used. This solubility measurement technique was also to be applied to canavalin. For the above reasons, and other difficulties associated with the application of the scintillation technique to canavalin (for a detailed account see the Final Report for NAG8-711), we have conducted the solubility study with equine serum albumin (ESA) instead. This protein, like other albumins, is a very useful plasma proteins. ESA is an extremely stable, clinically important protein due to its capability to bind and transport many different small ions and molecules [1]. The x-ray diffraction resolution of ESA has been determined to only 3.1 Å [2].

2. Determination of Useful Temperature Ramping Ranges

2.1 *Thermostated solution growth cell with cold sting*

The thermostated solution growth cell with cold sting (see Figure 1) was described in detail in earlier progress reports; see, in particular the Final Report for Grant NAG8-711. In short, it consists of a small thermostated growth chamber that has a sting attached to its bottom, the temperature of which is controlled separately. This cell was designed to utilize the temperature dependence of protein solubility for an efficient control of protein nucleation and crystal growth. By judiciously choosing the jacket temperature, T_J , and the sting temperature, T_S , one can limit the

area wherein crystals form initially, reduce the degree of supersaturation upon nucleation, and decrease the total number of crystals by selective dissolution. The interior of the growth chamber and, in particular, the sting region of its bottom can be viewed with a long focal length microscope. Crystal sizes can be monitored with a calibrated reticule in the microscope.

2.2 *Preparation of lysozyme solutions*

Lysozyme chloride from chicken egg white, grade VI (L2879, 90% protein, 5% NaCl) was purchased from Sigma Chemical Company. The protein was dissolved in deionized water at room temperature. The solution was filtered with a glass fiber filter using a Buchner funnel and vacuum flask and then with a Nalgene sterile (0.4 μm) filter. To remove salts from the protein solution, the filtrate was dialyzed against deionized water for a total of 29 hours. To obtain the desired buffering conditions, the protein was then dialyzed against 0.05 M acetate buffer, pH 4.5, 0.01% NaN_3 for a total of 60 hours. During this time, the buffer in the reservoir was periodically replaced with fresh solution. After the dialysis, the lysozyme solution was filtered using a Nalgene sterile (0.4 μm) filter and concentrated using an Amicon ultrafiltration apparatus. The pH of the protein solution was measured with an Orion SA 520 pH meter. The concentration of lysozyme was obtained from optical absorbance measurements at 280 nm with a Beckman DU-68 spectrophotometer using Beer's law and assuming $\epsilon_{1\%, 280\text{ nm}}=26.4$. The concentrated lysozyme solution was then stored at 4°C. In addition, stock solutions of 0.05 M acetate buffer, pH 4.5, 0.01% NaN_3 and of NaCl in the buffer were prepared and stored at 4°C. For each experiment, appropriate amounts of the stock lysozyme, buffer, and salt solutions were mixed. The resulting solution was centrifuged at 9,000-10,000 rpm in a Savant microcentrifuge to remove dust and/or particulate matter. The lysozyme concentration was subsequently determined from absorbance measurements. The sting cell experiments were carried out with solutions that contained 54 mg/ml lysozyme in 2.25% NaCl, 0.05 M acetate buffer, pH 4.5, 0.01% NaN_3 . Before a sting cell experiment, the solution was allowed to sit overnight at room temperature in order that any amorphous precipitate would settle and could be removed by centrifugation before placing the solution in the cell.

2.3 *Experiments and results*

The concentration of the lysozyme solution that, according to our solubility curves, corresponds to a saturation temperature of approximately 31°C was loaded into the cell. Then T_s and T_j were lowered to 20°C at the rate of 1°C per minute. After 26 hours at this temperature, no crystallites were seen, and both temperatures were lowered to 18°C at a rate of 1°C per minute. After 24 hours three small tetragonal lysozyme crystals were observed -- two crystals were on the surface of the growth sting, and one was on the wall of the jacketed region (see Figure 2). One crystal was 0.125 x 0.0625 mm, and the other two crystals were 0.0625 x 0.02 mm. T_s and T_j were then increased to 20.5°C over a two and a half hour period. Minimal growth occurred at

20.5°C overnight, so the temperatures were again decreased to 20°C. After six days at this temperature the crystals measured 0.331×0.125 mm and 0.291×0.083 mm, respectively. Thus, the average growth rates of these crystals at 20°C were 0.011×0.00047 mm/hour and 0.0015×0.00035 mm/hour. T_S and T_J were then lowered to 19.5°C. After 19 hours at this temperature, three new crystallites were detected. Twenty-one hours later, four more crystallites had formed (see Figure 3). The sizes of the two previously measured crystals were determined again. At 19.5°C these crystals had grown to 0.395×0.146 mm and 0.375×0.104 mm, corresponding to an average growth rates of 0.0016×0.0006 mm/hour and 0.0021×0.0006 mm/hour. To reduce the chance of further nucleation the temperatures were then increased to 20°C for one more week. The crystals reached sizes of 0.562×0.188 mm and 0.458×0.208 mm with average growth rates of 0.0010×0.0003 mm/hour and 0.0011×0.0006 mm/hour. These growth data are summarized in Fig. 4.

In order to investigate the dissolution behavior of these crystals, T_S and T_J were increased to 30°C over a period of two days. At this temperature slow dissolution occurred. The temperatures were further increased to 34.5°C, where all crystalline material seemed to dissolve. However, upon lowering both temperatures to 16°C at a rate of 1°C per minute, little crystallites covered all surfaces of the growth chamber after only five hours (see Figure 5). Then the temperatures were increased to 22°C where they were held overnight. At this temperature, further nucleation was not expected and only minimal growth was anticipated. The following day T_S was raised to 30°C and T_J to 34°C over a one hour period. Within thirty minutes at these temperatures, the crystals on the surface of the jacket began to dissolve. T_S and T_J were kept at 30°C and 34°C, respectively, for two days. By then, all crystalline material had dissolved.

Both temperatures were then lowered to 25°C for 48 hours. Following this, T_S and T_J were set to 17.5°C and 18.5°C, respectively, to determine the effect of a temperature gradient on nucleation. After only four hours many crystallites were detected on the surface of the growth sting, but only a few were present in the jacketed region of the cell.

This extended experiment with the lysozyme solution yielded much information. The findings can be summarized as follows:

- 1) As shown by Fig. 6 the crystals formed in this experiment were tetragonal. This was expected since nucleation occurred at 16°C to 18°C, where the supersaturation is about 5.4.. The aspect ratios of these crystals, however, are different from the tetragonal crystals that after nucleation at 10°C (supersaturation 27) were grown at the same temperature in an earlier experiment (see Fig. 7). Though Durbin and Feher [3] reported that the growth rates for the tetragonal lysozyme crystal faces {110} and {101} are concentration-dependent, which results in different crystal shapes depending on the growth conditions, habit dependence on nucleation conditions has not been described before.

2) As illustrated in Fig. 4, a temperature change of only 0.5°C can significantly increase the growth rates of lysozyme crystals. Also such a small temperature change can induce further nucleation.

3) This experiment also showed that upon waiting several hours between small temperature changes, nucleation will manifest itself by the formation of only a few crystals. If, however, larger undercoolings are immediately approached, the result is the formation of many crystals. Though this is well understood in the crystallization of inorganic materials, this insight has yet to be capitalized upon for nucleation control in protein crystallization. Furthermore, this experiment illustrated that a temperature gradient of a few degrees is sufficient to allow for both, spatially selective nucleation and dissolution.

Based upon these studies, and preliminary kinetics studies that were summarized in the Final Report of Grant NAG8-711, it is recommended that the **temperature stability** of at least 0.1°C and the possibility to establish **temperature gradients** of the order of $5^{\circ}\text{C}/\text{cm}$ should be provided for in future protein crystal growth apparatuses, if temperature control of nucleation and growth is desired. In addition, design requirements should include temperature ramping rates of order $2^{\circ}\text{C}/\text{hour}$. The temperature range required will, to some extent, depend on the specific protein considered.

Encouraged by the above results, we have begun work to grow protein crystals directly inside x-ray capillary tubes through temperature control. This will exclude the potentially damaging handling of the crystals during their transfer to the capillary before x-ray diffraction studies and, due to the small diameter of these tubes, will also lead to a reduction of convection during growth.

3. Solubility Measurements

3.1 *Scintillation technique*

Figure 8 shows a schematic of our experimental arrangement for the determination of temperature-dependent (protein) solubilities utilizing optical scintillation as a means of monitoring the nucleation and dissolution of crystallites. The working principle and the experimental components and of the system were described in detail the Final Report of Grant NAG8-711. In the following we will only report on the progress made towards semi-automation of the solubility determinations.

In order to overcome the limitations on experiment control imposed by the MacADIOS Manager data acquisition software package (supplied with the MacADIOS Model 411 hardware), we have developed our own QuickBASIC program. This program, which is used to store the photodiode and thermistor readings, smooth the data, display them on the computer screen, and execute incremental bath temperature changes, is listed in Table 1. Among other improvements,

the program periodically determines the time rate of change of the photodiode (scintillation) signal. This allows us to save considerable experiment time by initiating consecutive temperature changes at certain minimum slopes of the scintillation signal rather than waiting for a new equilibrium state to be reached after each temperature increment. Since the implementation of this program, semi-automated and reproducible solubility determinations have become routine. Further work will be concerned with a miniaturization of the system, employing fiber optics, to make this technique applicable to small quantities when expensive proteins are to be studied.

3.2 *Preparation and characterization of equine serum albumin (ESA) solutions*

ESA (A5280, Lot 66F9381, Cohn's Fraction V, essentially fatty acid free, 99% albumin) was purchased from Sigma Chemical Company. Before experimenting with ESA in the scintillation cell, we performed numerous crystallization experiments in order to characterize the batch of protein, to reproducibly obtain crystals, and to determine the range of conditions (choice of precipitating agent, salt concentration, protein concentration, buffer, pH) that should be studied with the scintillation technique. Based upon these experiments, ESA's solubility was studied using the following conditions: 40-50% ammonium sulfate, 0-60 mg/ml ESA, 0.024 mg/ml NaN_3 , 0.05 M acetate buffer, pH 5.5. These conditions resulted in the formation of hexagonal crystals like those reported in [2]. It should be emphasized that these conditions require much higher concentrations of salt than our studies with lysozyme (see Final Report for NASA Grant 8-711). Such high concentrations are often found necessary for the crystallization of proteins with retrograde solubility. As we will show below, ESA is, indeed, a protein with strong retrograde solubility. Furthermore, since a protein is generally less soluble at or near its isoelectric point, we chose to study this system using a buffer of pH 5.5. ESA has an isoelectric point of approximately 4.9 [4].

For a series of experiments, a stock ESA solution was prepared by dissolving the protein in 0.05M acetate buffer, pH 5.5, 0.01% NaN_3 , centrifuging the solution at 9,000 rpm, and filtering with a 0.2 μm syringe filter. The ESA stock solution was stored at 4°C in a capped, sterile, 5 ml test tube. Each batch of ESA stock was kept for no longer than two weeks. This procedure was followed based on work done in [5] which verified the microheterogeneity of the solution significantly increased after such a time period.

Before preparation of the solutions to be studied with the scintillation technique, the ESA stock solution was subjected to isoelectric focusing (IEF) and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) in order to evaluate the purity of the albumin. The materials used were Pharmacia IEF/PAGE Ampholine PAGplates, pH 3.5-9.0 and Pharmacia SDS ExcelGels (PA 8-18). The three most probable contaminating protein components (based upon the isolation and purification methods used by Sigma Chemical Company in the albumin preparation)

were the α , β , and γ globulins with isoelectric points, respectively, at 4.9, 6.3, and 6 to 7 [4]. The published isoelectric point of ESA is 4.9 [4], and its molecular weight is 67,500 [6].

The IEF/PAGE experiments with the ESA solutions resulted in multiple bands with isoelectric points between 4.5 and 5.3. These IEF/PAGE experiments indicated that only α -globulin possibly contaminated our ESA stock. Moreover, the SDS/PAGE experiments resulted in only one band of an approximate molecular weight of 67,000. Since the SDS/PAGE showed the presence of no other bands, particularly in the region of α -globulin molecular weight (approximately 147,000 [7]), we conclude that our ESA was essentially free from contaminating proteins. Therefore, the multiple banding of the ESA samples is, at this point in time, believed to be due to the microheterogeneity of the albumin itself. Possible sources of microheterogeneity include bound non-protein impurities, variations in the amide content, variations in the amino acid composition of the molecules, variations in disulfide pairings, and differences in secondary or tertiary structure [5]. It is also possible that ESA reacts with the ampholytes in the polyacrylamide gel, and these interactions may cause the multiple banding around the isoelectric point of the protein [8]. It has long been known that ESA does not consist of a single chemical individual as judged by solubility or dielectric studies even though it appears homogeneous as evidenced by molecular weight determinations and its electrophoretic mobility [1]. The ESA stock material will be analyzed further on a Pharmacia Ampholine PAGplate, pH range 4.0-6.5.

For each scintillation experiment appropriate volumes of stock protein, salt (in buffer), NaN_3 (in buffer), and buffer solutions were mixed. The ESA concentration was determined by absorbance measurements at 280 nm ($\epsilon_{1\%}^{1\text{cm}} = 0.515$) with a Beckman DU-68 spectrophotometer before and after each experiment. The pH of the protein solution was measured before and after each run using an Orion SA 520 pH meter and pH micro-probe. Although buffer with pH 5.5 was used, the pH of all protein solutions after each solubility experiment was 6.3.

3.3 Solubility of equine serum albumin

Figures 9-11 show typical data obtained from the ESA scintillation solubility studies. Figure 12 summarizes the temperature dependence of hexagonal ESA solubility for three different ammonium sulfate concentrations, i.e. 43%, 45%, and 47%. These curves reveal two interesting features. Most importantly, ESA possesses retrograde solubility, i.e. nucleation and dissolution occur upon heating and cooling the solution, respectively. We found later that the retrograde solubility of ESA had been qualitatively observed by McMeekin in 1939 [6]. Furthermore, the solubility decreases with increasing ammonium sulfate concentration. Though it is often undesirable to grow crystals in the presence of high salt concentrations, because of the potential complications of interface kinetics steps, with ESA it is necessary to use at least 30% ammonium sulfate to drive the crystallization process. For example, in one experiment we attempted to

nucleate a 250 mg/ml ESA, 5% ammonium sulfate solution, though without success even after 36 hours at 40°C and seeding.

It is also noteworthy that, by comparison with our earlier work with lysozyme, ESA showed somewhat slower dissolution kinetics. Furthermore, as dissolution of ESA crystallites began, "wispieness" (swirling of the crystallites in a ribbon-like formation as the material in the cell was stirred) occurred, causing noise in the photodiode signal. This problem was resolved by averaging 500 photodiode (microsecond) signals over a few seconds.

4. Summary

In short, we have:

- determined temperature stability, gradient and ramping rate requirements useful for protein crystallization;
- shown that nucleation followed by growth without further nucleation can be accomplished through appropriate temperature programming. This can be performed most efficiently if based upon predetermined solubility data; and
- determined the solubility of equine serum albumin as a function of temperature at three different ammonium sulfate concentrations.

5. References

- [1] E. J. Cohn, *The Plasma Proteins* (1941) 395.
- [2] R. J. McClure and B. M. Craven, *J. Mol. Biol.* 83 (1974) 551.
- [3] S. D. Durbin and G. Feher, *J. Crystal Growth* 76 (1986) 583-592.
- [4] E. J. Cohn, et al, *J. Am. Chem. Soc.* 72 (1950) 465.
- [5] J. Janatova, *J. Med.* 5 (1974) 149.
- [6] T. L. McMeekin, *J. Am. Chem. Soc.* 61 (1939) 2884.
- [7] E. J. Cohn, et al, *J. Am. Chem. Soc.* 62 (1940) 3386.
- [8] R. K. Scopes, Protein Purification Principles and Practice, Second Edition, Springer-Verlag New York Inc., 1987.

6. Presentations of Research Results under this Grant

T. A. Nyce; Temperature Control of Nucleation and Growth of Protein Crystals; presentation at New Developments in Protein Crystal Growth Workshop, Gulf Shores, Alabama, May 11-14, 1990.

L. Monaco, N.-B. Ming and F. Rosenberger; High Resolution Microscopy of Lysozyme-Solution Interfaces; poster at New Developments in Protein Crystal Growth Workshop, Gulf Shores, Alabama, May 11-14, 1990.

T. A. Nyce; Temperature Control of Nucleation and Growth of Protein Crystals; presentation at Eighth American Conference on Crystal Growth, Vail, Colorado, July 15-20, 1990.

F. Rosenberger; Protein Crystal Growth; invited presentation at NASA Microgravity Fluids Workshop; Cleveland, Ohio, August 7-9, 1990.

7. Figure Captions, Figures and Table

Figure 1. Schematic of thermostated cell with growth sting.

Figure 2. View of bottom of growth chamber of the thermostated cell with three lysozyme crystals nucleated from a 54 mg/ml solution at 18°C. The inner circle outlines the boundary of the sting.

Figure 3. Growth of crystals in Figure 2 and new crystals that formed at 19.5°C.

Figure 4. Growth data for two crystals in the thermostated cell with growth sting.

Figure 5. Lysozyme crystallites nucleated over entire surface of the growth cell from a 54 mg/ml solution at 16°C. The inner circle outlines the boundary of the sting.

Figure 6. Tetragonal lysozyme crystal, nucleated at 18°C, grown at 20°C.

Figure 7. Tetragonal lysozyme crystals, nucleated at 10°C, grown at 20°C.

Figure 8. Schematic of system for temperature controlled protein crystallization and dissolution with detection of crystallites by optical scintillation.

Figure 9. Equine serum albumin dissolution with decreasing temperature monitored by scintillation signal. ESA concentration 34.5 mg/ml, 43% ammonium sulfate, pH=6.3.

Figure 10. Equine serum albumin dissolution with decreasing temperature monitored by scintillation signal. ESA concentration 48.5 mg/ml, 45% ammonium sulfate, pH=6.3.

Figure 11. Equine serum albumin dissolution with decreasing temperature monitored by scintillation signal. ESA concentration 24.0 mg/ml, 47% ammonium sulfate, pH=6.3.

Figure 12. Solubility of hexagonal equine serum albumin versus temperature, scintillation method.

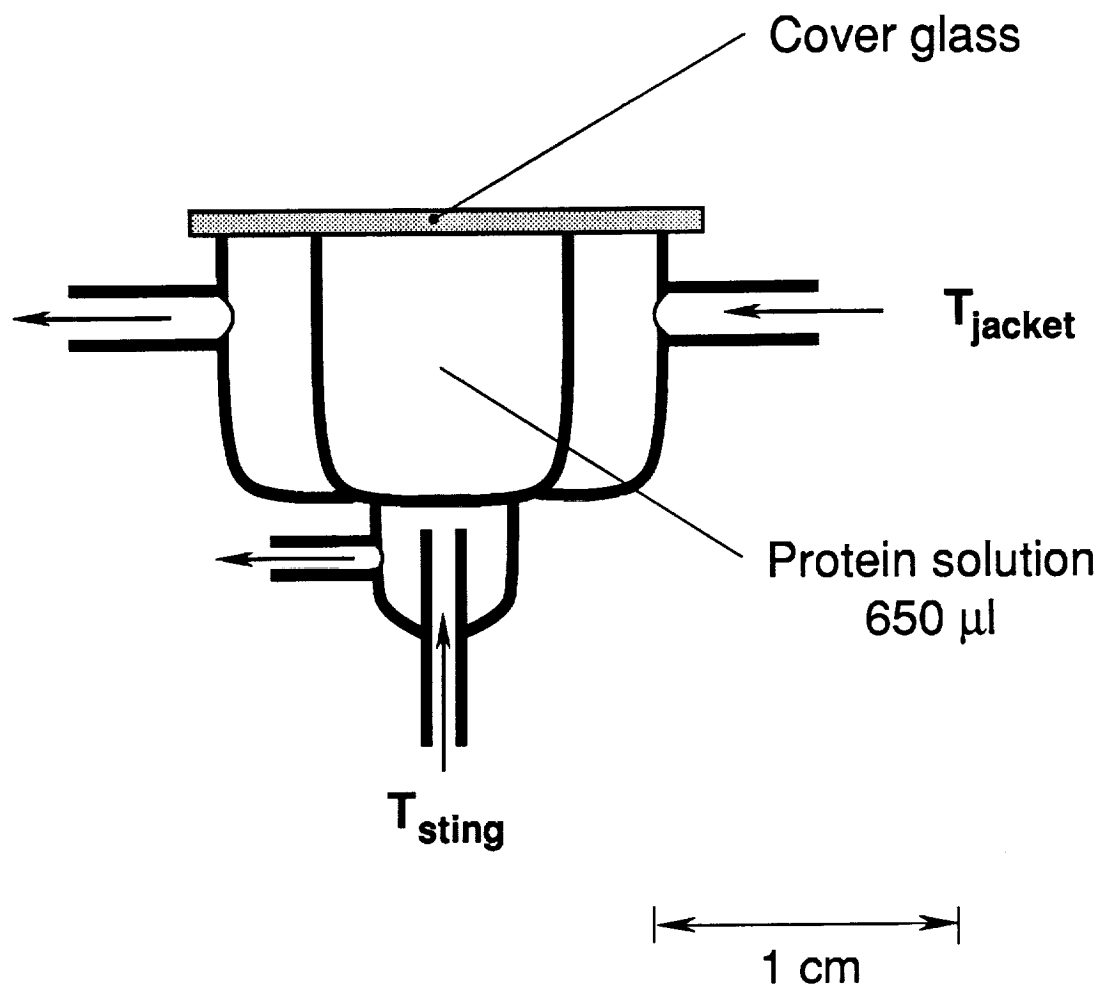


FIG. 1



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1 mm

FIG. 2



1 mm

ORIGINAL PAGE
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FIG. 3

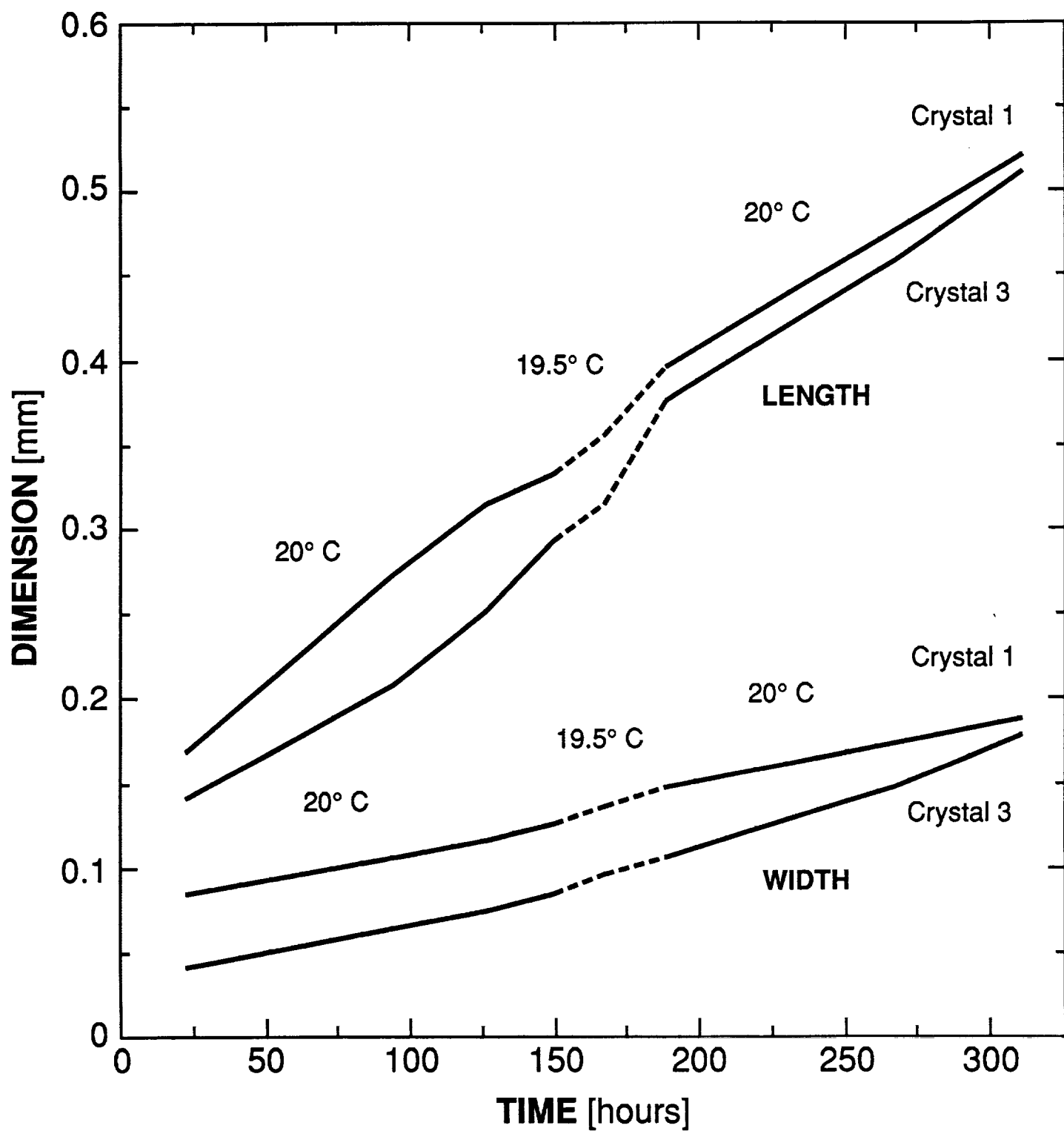


FIG. 4

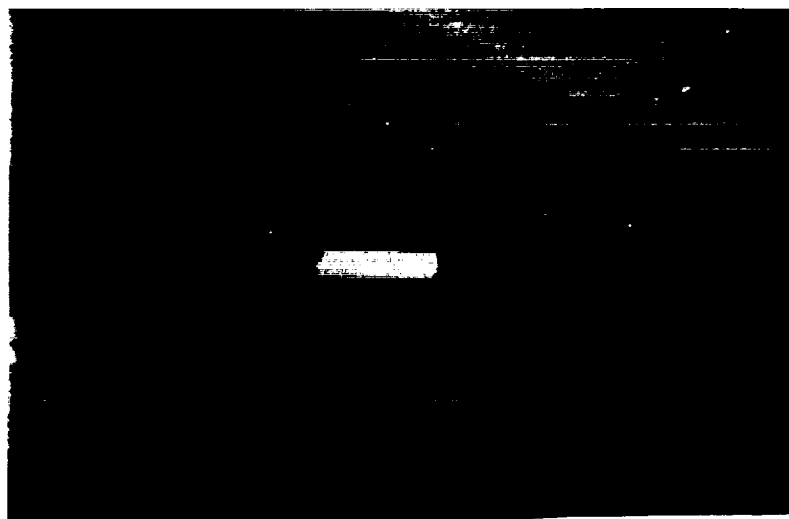


1 mm

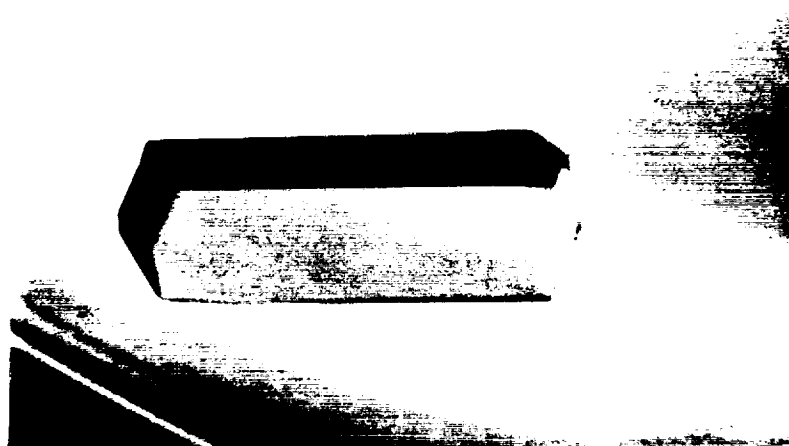
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FIG. 5

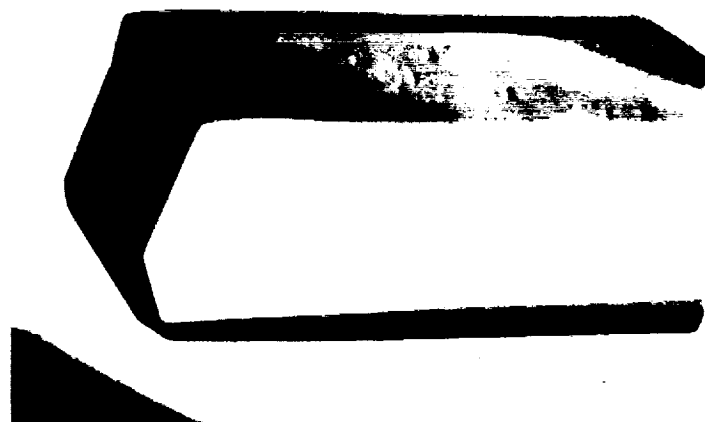
4 days



11 days



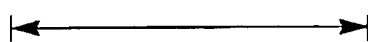
16 days



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FIG. 6



0.2 mm

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FIG. 7

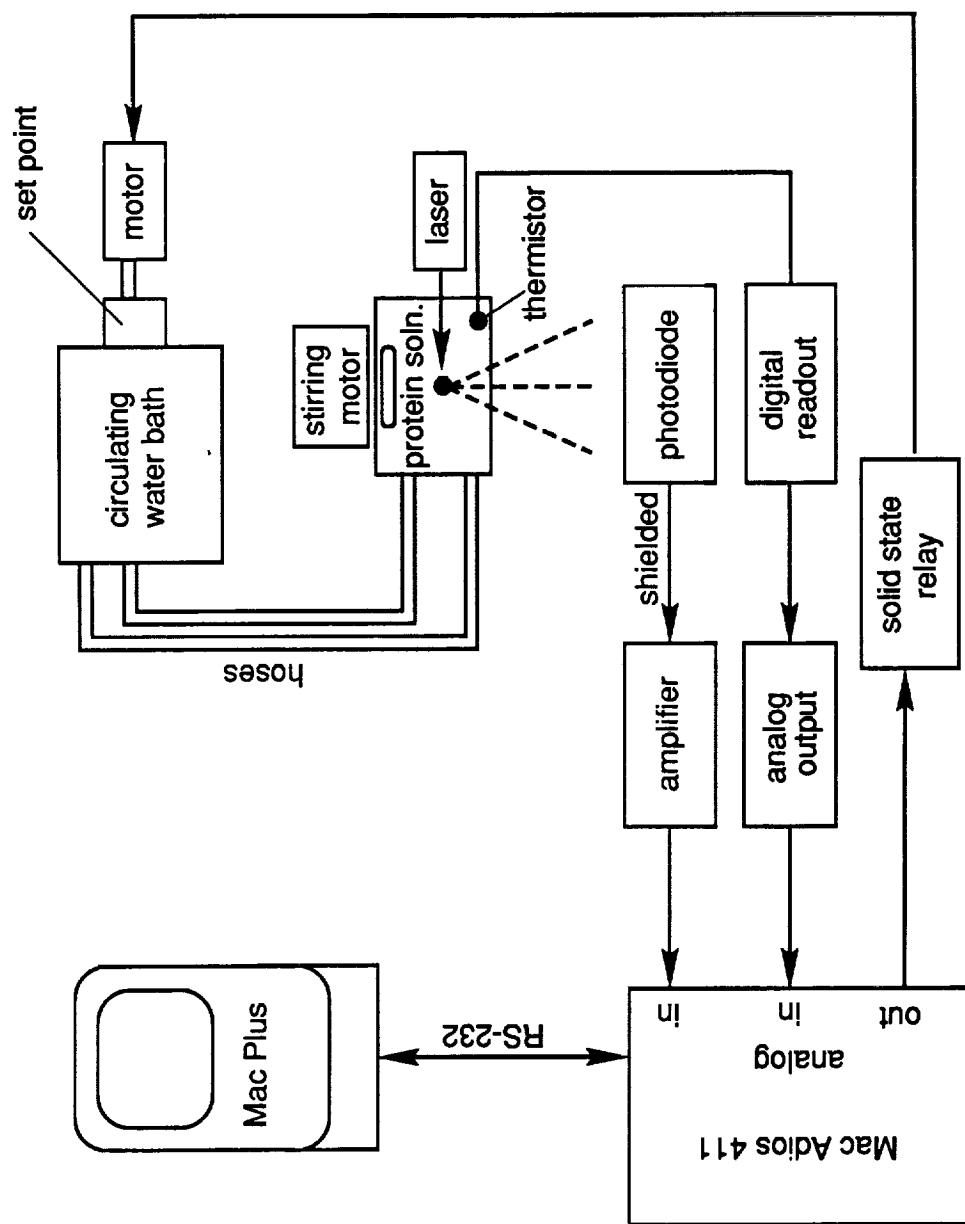


FIG. 8

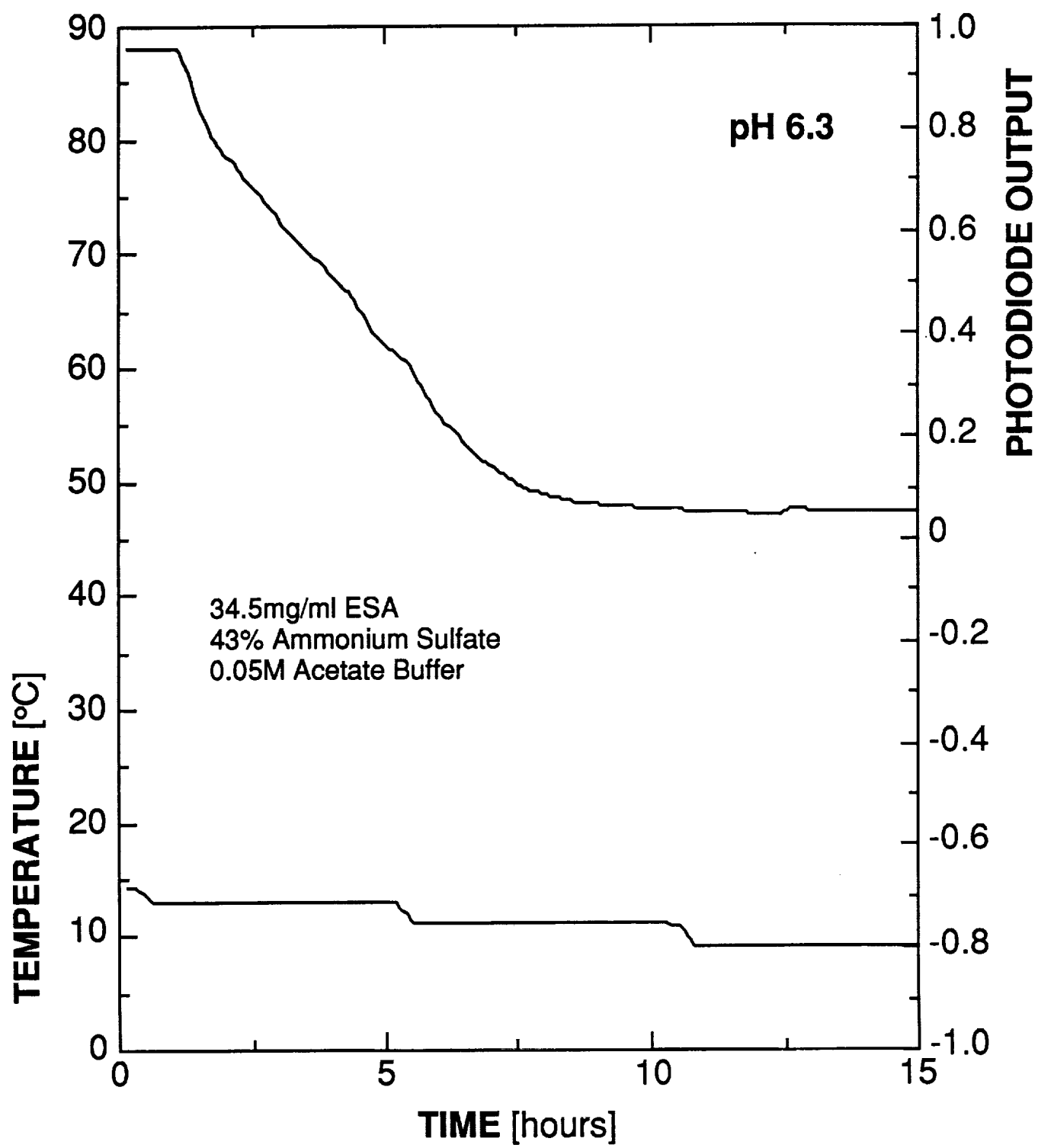


FIG. 9

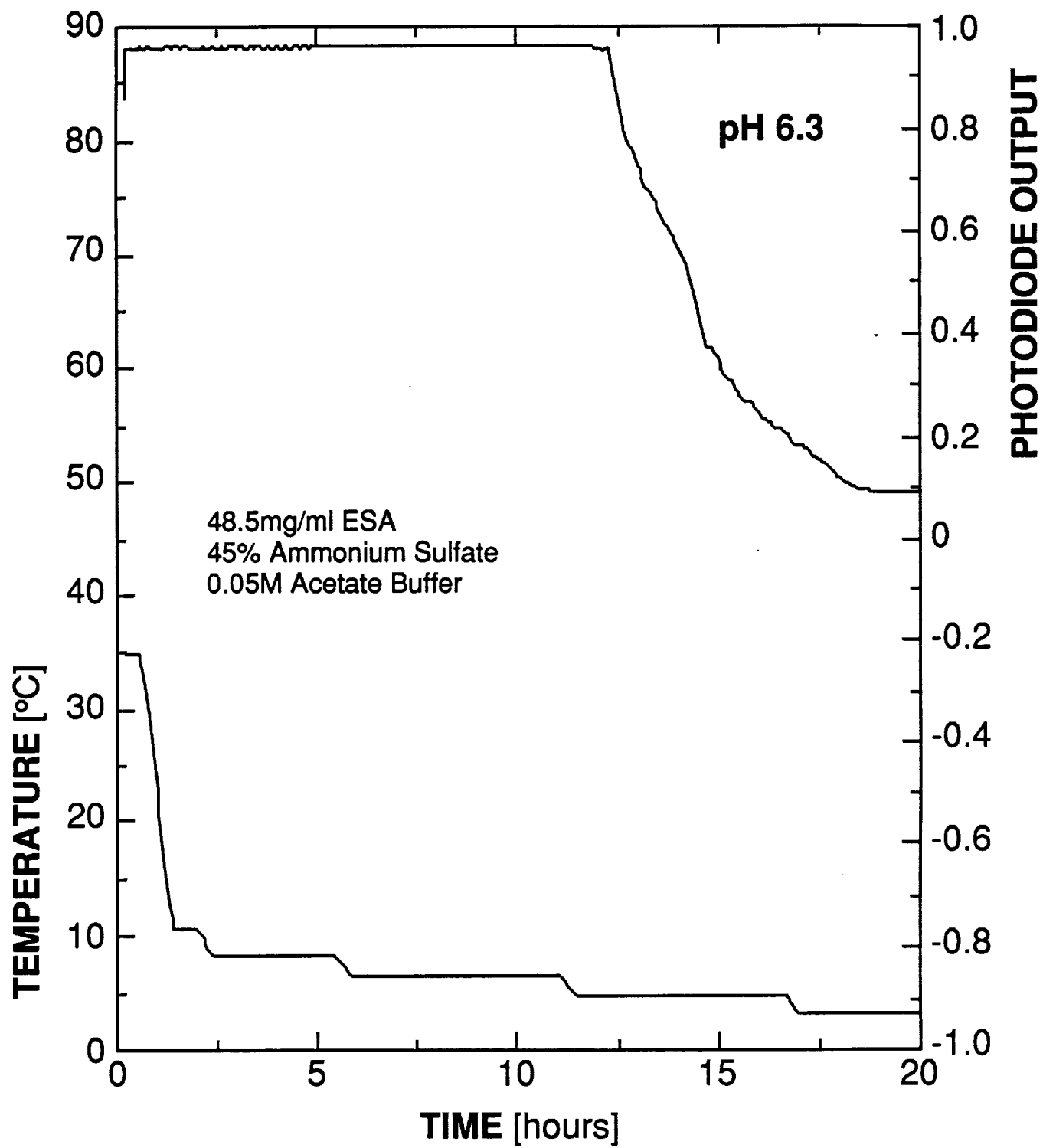


FIG. 10

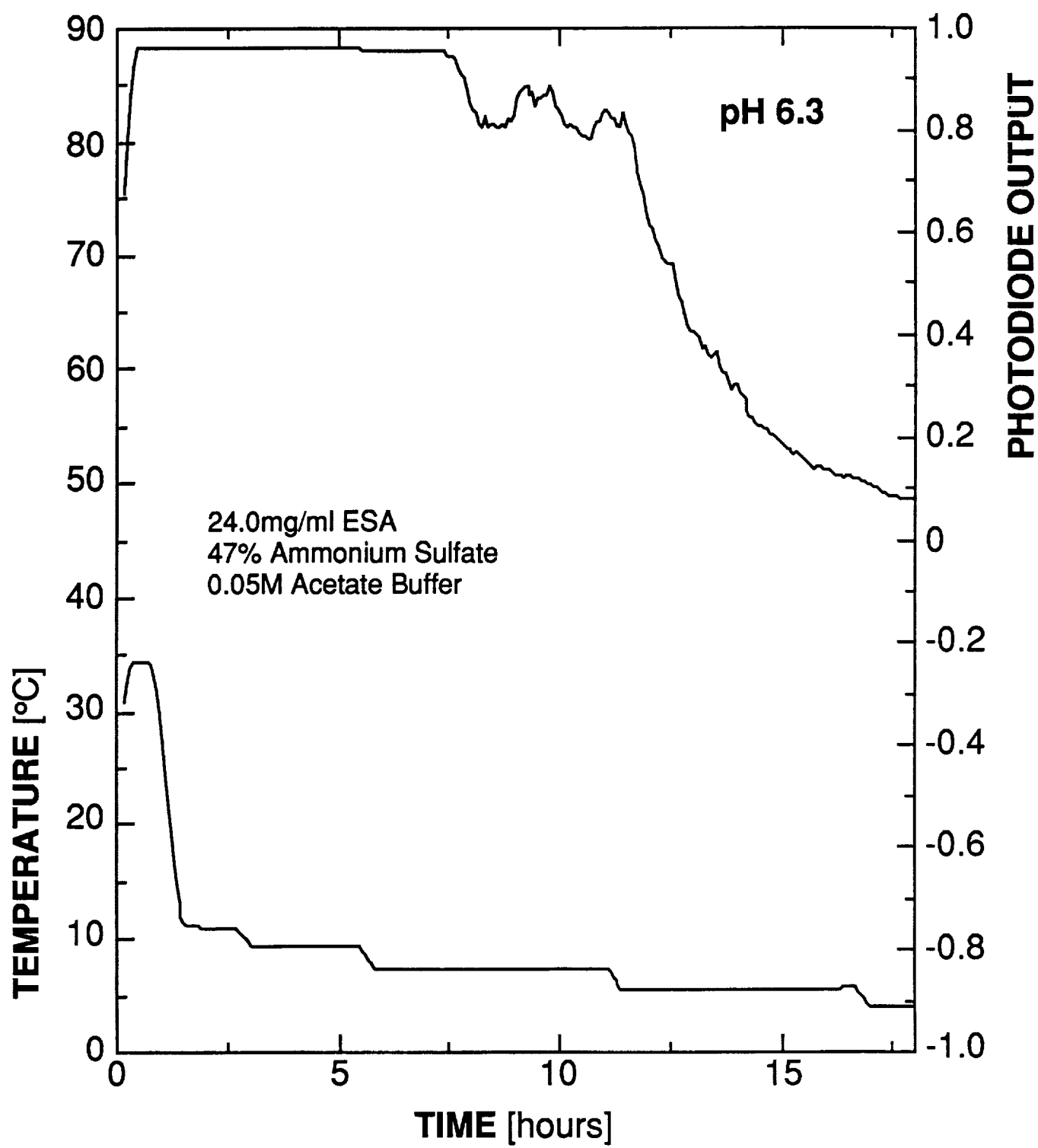


FIG. 11

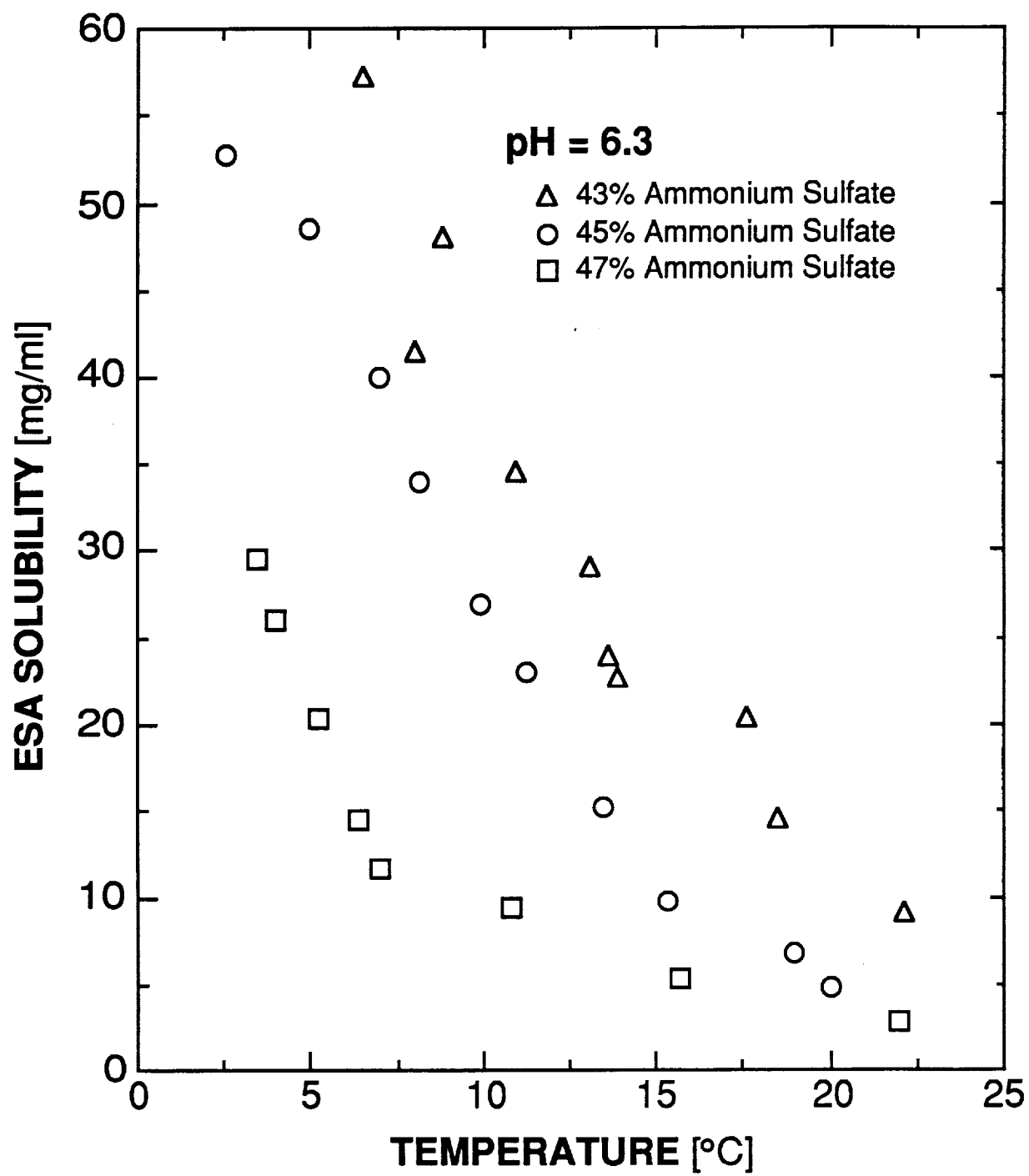


FIG. 12

TABLE 1: SAMPLE BASIC CONTROL PROGRAM FOR SCINTILLATION SOLUBILITY MEASUREMENT TECHNIQUE

```

LIBRARY "411Drivers Interface" (for using MACADIOS 411 Drivers library)
LIBRARY "DataManipulation Interface" (for using MACADIOS DataManipulation library)
10 CALL MAINIT (calls a MACADIOS function)
20 OPEN "clip:" FOR OUTPUT AS#1 (data will be stored in clipboard when END statement is reached)
30 DIM T%(600), TEMP%(600) (dimension statement will allow 600 temperature data points)
40 DIM d%(600), diode%(600) (dimension statement will allow 600 photodiode data points)
50 LET DSUM!=0 (definition statement)
60 LET TSUM!=0 (definition statement)
70 ER%=0 (definition statement)
75 LET j=0: k=0 (definition statement)
80 INPUT "Enter desired value of SETUP (microseconds)"; setup (timing of cycles)
100 INPUT "Enter desired value of MEASURE (microseconds)"; measure (timing of cycles)
120 PRINT "CYCLES will be set to (seconds): "; (measure+setup)/1000000& (time per cycle)
130 n%=19999 : T=2*(n%+1) : l%=setup/T : m%=(measure-T)/(T*l%+T) (timing formulas)
133 INPUT "Would you like the temperature increments to be .5, 1, 1.5, or 2 (°C)?";
      tempchange (allows user to choose temperature increment for experiment)
134 IF tempchange=.5 THEN LET a=25:b=10:c=19999:d=1 (information directing the
135 IF tempchange=1 THEN LET a=25:b=20:c=19999:d=1 output signal to the motor
136 IF tempchange=1.5 THEN LET a=25:b=31:c=19999:d=1 to change the bath's set point:
137 IF tempchange=2 THEN LET a=25:b=42:c=19999:d=1 based on chosen increment)
140 CLS (clears screen)
150 INPUT "THE BEGINNING DATE OF THIS SCINTILLATION CELL RUN IS ", MDY$
160 INPUT "THE SOLUTION IS ", SOLUTION$
      (allows user to input information about experimental conditions)
170 CALL grid(2,150,250,0,610,11,10,61,10) (grid for temperature data display)
180 CALL grid(2,30,130,0,610,11,10,61,10) (grid for photodiode data display)
190 FOR index%=0 TO 599 STEP 1 (sets number of cycles)
200 CALL msinit (l%,m%,n%,1) (sets timing according to SETUP and MEASURE values)
210 CALL swait (waits the appropriate amount of time before taking data points for the cycle)
220 CALL msinit (4,1,1000,1) (sets timing for the collection of data)
230 CALL AINX(1,0,500,VARPTR(ER%),VARPTR(d%(0)),VARPTR(T%(0)),0,0,0,0,0,0)
      (specifies the number of points (500) for each AINX that will be collected during a cycle)
240 CALL INTEG (VARPTR(T%(0)),0,499,VARPTR(TSUM!),-2)
      (sums the 500 temperature data points)
250 CALL INTEG (VARPTR(d%(0)),0,499,VARPTR(DSUM!),-2)
      (sums the 500 photodiode data points)
260 LET DIODE%(index%) = DSUM!/500 (averages the 500 photodiode points)
270 LET TEMP%(index%) = TSUM!/500 (averages the 500 temperature points)
280 IF DIODE%(index%)<1000 THEN GOTO 310
      (directs program to Line 400 if photodiode data point is less than 1000 in order to bypass
      temperature changes after all material has dissolved))
290 IF DIODE%(index%)>9000 AND FIX(index%/15)=index%/15 THEN GOSUB
      SATURATION (every 55 minutes directs program to subroutine SATURATION if diode is
      saturated)
300 IF DIODE%(index%)<9000 AND index%>10 AND FIX(index%/10)=index%/10 THEN
      GOSUB SLOPE (every 40 minutes directs program to subroutine SLOPE if material has started
      to dissolve)
310 IF index%>0 AND FIX(index%/25)=index%/25 THEN GOSUB DISK

```



```

        (every 1.5 hours directs program to subroutine DISK to save data)
320 PRINT #1, DIODE%(index%), TEMP%(index%) (prints data to clipboard)
330 CALL plot(0,150,250,0,VARPTR(TEMP%(0)),5000,0,index%,1,0,1,0)
        (plots temperature data on the displayed temperature grid)
340 CALL plot(0,30,130,0,VARPTR(DIODE%(0)),10000,0,index%,1,0,1,0)
        (plots photodiode data on the displayed photodiode grid)
350 NEXT index% (indicates the beginning of the next cycle; returns to the beginning of the loop, Line 190)
360 diskerrorcode%=30 (definition statement for bsave command)
370 CALL bsave("programs:", "diode", VARPTR(DIODE%(0)), type%, 1200&,
        VARPTR(diskerrorcode%)) (saves photodiode data to disk upon completion of all cycles)
380 CALL bsave("programs:", "temp", VARPTR(TEMP%(0)), type%, 1200&,
        VARPTR(diskerrorcode%)) (saves temperature data to disk upon completion of all cycles)
390 END (end of program)

```

DISK: (subroutine that saves data to disk as directed by Line 400)

```

diskerrorcode%=30 (definition statement)
a$="programs:";b$="diode";c$="temp" (definition statement; must use $ to prevent error message
        "String Formula Too Complex" after the second time the statement is executed)
CALL bsave(a$,b$,VARPTR(diode%(0)),type%,1200&,VARPTR(diskerrorcode%))
        (saves photodiode data to disk)
CALL bsave(a$,c$,VARPTR(TEMP%(0)),type%,1200&,VARPTR(diskerrorcode%))
        (saves temperature data to disk)
RETURN (returns program to Line 410)

```

SATURATION: (subroutine that directs a temperature change if the diode is saturated as directed by Line 290)

```

CALL aout(0,0,0,2500) (sends output signal via solid state relay to motor connected to bath set point)
CALL msinit(25,42,19999,1) (sets length of time (thus, the temperature change) signal will be sent to motor)
CALL swait (waits the amount of time while the signal is sent to motor)
CALL aout(0,0,0,0) (turns off the output signal to the motor)
RETURN (returns program to Line 300)

```

SLOPE: (subroutine that directs incremental temperature changes based on the slope of the photodiode data points when the material has started to dissolve; directed by Line 300)

```

LET j=(diode%(index%-9)+diode%(index%-8)+diode%(index%-7)+diode%(index%-
        6)+diode%(index%-5))/5 (averages photodiode points from last five minus five cycles)
LET k=(diode%(index%-4)+diode%(index%-3)+diode%(index%-2)+diode%(index%-
        1)+diode%(index%))/5 (averages photodiode points from last five cycles)
LET l=ABS((j-k)/5) (calculates an "average slope" of the last ten photodiode data points)
IF l<12 THEN GOSUB INCREMENT (if the slope of the last ten photodiode data points is less than
        twelve, the program is directed to the subroutine INCREMENT)
RETURN (returns program to Line 310)

```

INCREMENT: (subroutine that directs incremental temperature changes by sending an output signal via solid state relay to the motor connected to the bath set point; based on the SETUP and MEASURE specified by the user at the beginning of the experiment)

```

CALL aout(0,0,0,2500) (sends output signal via solid state relay to motor connected to bath set point)
CALL msinit(a,b,c,d) (sets length of time the output signal will be sent to the motor; based on the SETUP
        and MEASURE values specified by the user)
CALL swait (waits the amount of time while the signal is sent to motor)
CALL aout(0,0,0,0) (turns off the output signal to the motor)
RETURN (returns program to Line 310)

```